IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: IKEHARA, et al.
Serial No.: 09/380,579 Group Art Unit: 1644
Filed: September 7, 1999 Examiner: Michail A. Belyavskyi, Ph.D
For: Immunotolerance inducer

DECLARATION

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Susumu IKEHARA, hereby declare that:

1. I am one of the inventors of the instant invention and I am fully familiar with the subject matter thereof.
2. I received my M.D. from Kyoto University in 1968.
   I received my Ph.D from Kyoto University in 1977.
3. I graduated from the School of Medicine, Kyoto University in 1967.
   I graduated from the Postgraduate School of Medicine, Kyoto University in 1975.
   I was Visiting Investigator of the Memorial Sloan-Kettering Cancer Center (President Robert A. Good) in New York, 1978-1981.
   Professor of Dept. of Pathology, Kansai Medical University, 1985~present
   Director of Transplantation Center, Kansai Medical University, 1998~2002
   Director of Regeneration Research Center for Intractable Diseases, 2001~present
   Director of Center for Cancer Therapy, 2003~present.

The experiments described below were carried out under my general direction and supervision.

EXPERIMENTAL DATA

(1) Purpose of Experiment

According to the Experiment shown here, it is shown clearly that the method of this invention can apply to tissue/organ allografts other than the skin in a mouse. Further, the Experiment shows that the method of this invention would be applicable to humans.

(2) Materials and Methods

Recipient and Donor

Female C57BL/6 (B6, H-2^b), (BALB/$_c$ × DBA/2) Fl (CDF1, H-2^d), and C3H/HeN (H-2^k)

mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Donor mice were used at the age of 7-8 weeks, and recipient mice were used at the age of 10-12 weeks.

Irradiation

Mice were exposed to 7Gy (1.05 Gy/min) from a ^{137}Cs source (Gammacell 40 Exactor, Nordion, International Inc, Kanata, Ontario, Canada).

Bone Marrow Transplantation and Organ Allografts

BMCs were collected from the femurs and tibias by flushing. T cells were eliminated by treating the BMCs with anti-Thy 1.2 mAbs (F7D5, Olac, Bicester, England) plus complement. Briefly, Bone marrow cells (BMCs) were incubated with anti-Thy 1.2 mAb at 4℃ for 30 min. They were then washed and resuspended in guinea pig complement at a 1/16 dilution in RPMI (1×10^7 cells/ml in diluted guinea pig complement) at 37℃ for 40 min. The BMCs were then washed twice and resuspended in RPMI at an appropriate concentration for injection of 0.5 ml of final volume per mouse. BMCs from donor mice were injected via P.V. Briefly, the mice were anesthetized with pentobarbital. Donor cells (3×10^7 in 0.3 ml of RPMI 1640 medium) were injected through the superior mesenteric vein using a 27-gauge needle. After the injection, hemostasis was secured by gentle pressure with a cotton-wool swab. The skin grafting was performed on the same day after the P.V. injection. Full-thickness skin grafts (1 cm × 1.5 cm) were harvested from the dorsal wall of the donor, from which the hair had been completely removed by plucking and using depilatory. Skin grafts were sutured to the graft beds on the thoracic wall using 6-0 nylon and covered with Vaseline, gauze, and protective tape. The first inspection of the skin graft was performed 21 days later, followed by inspections twice a week thereafter. Because the graft rejection started with loss of hair and culminated in necrosis of the graft skin, the graft was considered to have been rejected when no normal epithelium could be found on the graft beds. The grafting procedure for the fetal pancreas and adrenal glands was as follows; Organs were grafted into recipients. Recipients were anesthetized with an intraperitoneal injection of somnopentyl (0.1 mg/g body weight). Light ether anesthesia was used, if necessary, during the operation. A vertical incision was made in the lumbar region and the underlying fetal pancrea or adrenal gland then gently pulled out of the abdomen. A longitudinal incision was made in the renal capsule. The edge of the incised capsule was lifted up with fine forceps, and the fetal pancrea or the adrenal gland placed under the capsule and then pushed away from the incision. The fetal pancrea or the adrenal gland was replaced within the retroperitoneal cavity and the

abdomen muscle layer and skin incision was closed with silk sutures. The time required for engraftment of a single graft was about 10 min.

(3) Result

The B6 mice that had been treated with 7 Gy irradiation plus P.V. injection of CDF1 mouse BMCs accepted the CDF1 mouse skins for more than 1 year after the treatment (Fig. 1a). The B6 mice that had accepted the primary CDF1 mouse skins were further engrafted with CDF1 mouse skins 4 months after the treatment (7 Gy plus P.V.). As shown in Figure 1b, the secondary skins were also accepted by the chimeric mice, although the chimeric mice rejected the third-party C3H skins within 3 weeks (data not shown). Both skins were accepted for more than 5 months.

Firther, we grafted the pancreas or adrenal glands under the renal capsules. As shown in Figure 1 (c and d), the pancreas (both exocrine and endocrine glands) and adrenal glands were also accepted by recipients treated with 7 Gy plus P.V. injection of CDF1 mouse BMCs without recourse to immunosuppressants.

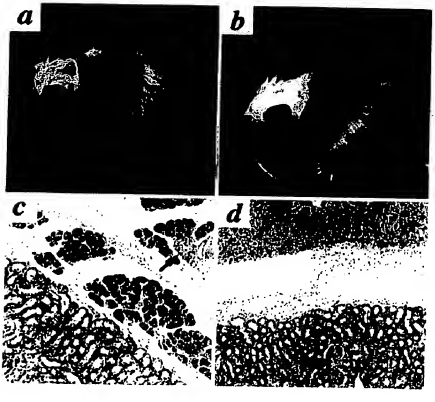

Figure 1.: Acceptance of organ allografts in recipients treated with 7 Gy plus P.V.
(a) The B6 mouse treated with 7 Gy plus P.V. injection of CDF1 mouse BMCs accepted

the CDFI skin for 1 year after the treatment (n=17).
(b) The B6 mouse treated with 7 Gy plus P.V. injection of CDF1 mouse BMCs accepted not only the primary skin but also the secondary skin (n=5).
(c) The recipient B6 mouse treated with 7 Gy plus P.V. injection of CDF1 mouse BMCs accepted the CDF1 mouse pancreas under the renal capsule for 1 year after the treatment (n=5). It should be noted that both exocrine and endocrine (arrow) glands can be seen (n=5).
(d) The mouse treated with 7 Gy plus P.V. injection of CDF1 mouse BMCs accepted the CDF1 mouse adrenal gland under the renal capsule for 1 year after the treatment (n=5). These figures consist of representative data from more than five experiments.

(4) Discussion

To examine whether this strategy is applicable to all organ allografts, we grafted the pancreas or adrenal glands under the renal capsules in addition to skin. As shown in Figure 1 above, not anly the skin but also the pancreas and the adrenal glands were found to be accepted by recipients treated with 7 Gy plus P.V.

We have carried out allogeneic organ transplantation research using mice as described above, and found that full chimerism (but not mixed chimerism) is essential to the acceptance of organ allografts for a long duration without using immunosuppressants.

It is well known that the most difficult tissues/organs (which are susceptible to rejection) are the skin and pancreas (pancreatic islets), since major histocompatible complex (MHC) class II molecules are highly expressed in these tissues; cytotoxic T-lymphocytes can easily recognize these molecules and kill the target cells. Therefore, if a new strategy is to be proved useful for skin and islet transplantation, it is generally accepted that this strategy could be applicable to all organ/tissue transplantation.

Further, recent methodologies for bone marrow transplantation (BMT) and/or organ transplantation have been established, based on a great quantity of experimental data mainly using mice and rats, since it is very difficult to use non-human primates such as monkeys in transplantation research. We have shown that our strategy is enough to be applicable to mice. Based on these findings, it is conceivable that our strategy would be applicable to humans and it is believed that we could provide evidence that our strategy would become a valuable transplantation method.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 5/18/2007

Susumu IKEHARA